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(54) Title: METHODS FOR IMPARTING DESIRABLE PHENOTYPIC TRAITS, INCLUDING DROUGHT, FREEZE, AND HIGH SALT TOLERANCE AND METHODS FOR INCREASING SEED PRODUCTION

(57) Abstract: Methods are provided for imparting desirable pohenotypic traits to transgenic plants, among them being increased tolerance to external stresses such as drought, freezing temperatures, high salt conditions, and the like. In addition, the present invention is directed toward methods for increasing the yield of seeds from plants by using the pollen from a transgenic plant transformed to overexpress a vacuolar proton-pumping pyrophosphatase to fertilize plants, and to the pollen of transgenic plants itself.

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TITLE OF THE INVENTION

METHODS FOR IMPARTING DESIRABLE PHENOTYPIC TRAITS, INCLUDING DROUGHT, FREEZE, AND HIGH SALT TOLERANCE AND METHODS FOR INCREASING SEED PRODUCTION

FIELD OF THE INVENTION

[0002] The present invention relates, in general, to methods of imparting desirable phenotypic traits in plants transformed with exogenous nucleotide sequences. More specifically, the present invention concerns imparting the ability to withstand external stresses such as drought, extended exposure to sub-freezing temperatures, and high salinity on plants through transformation with exogenous nucleotides encoding a tonoplast pyrophosphate-driven H+ pump. The present invention also concerns methods for increasing the production of seeds in plants by using pollen from genetically-altered plants to fertilize wild type or transgenic plants. The present invention also relates to the pollen produced by genetically altered plants.

BACKGROUND OF THE INVENTION

[0003] The prospects for feeding humanity as we enter the new millennium are formidable. Given the every increasing world population, it remains a major goal of agricultural research to improve crop yield. Until recently crop and plant improvements

depended on selective breeding of plants having desirable characteristics. Such selective breeding techniques, however, were often less than desirable as many plants had within them heterogeneous genetic complements that did not result in identical desirable traits to their parents.

[0004] Recently, advances in molecular biology have allowed mankind to manipulate the germplasm of animals and plants. Genetic engineering of plants entails the isolation and manipulation of genetic material (typically in the form of DNA or RNA) and the subsequent introduction of that genetic material into a plant or plant cells. Such technology has led to the development of plants with increased pest resistance, plants that are capable of expressing pharmaceuticals and other chemical, and plants that express beneficial traits. Advantageously such plants not only contain a gene of interest, but remain fertile and often desirably to pass the gene on to its progeny.

[0005] One particular area of interest of late has been the development of plants with improved production of seeds. Improving the yield in the production of seeds from cultivars, such as rice, canola, wheat, corn, and sunflower, can increase food production for animal and human consumption. Also, improved seed yield can have economic benefits by reducing the costs associated with producing seed for farming.

[0006] The yield of a plant crop may be improved by growing transgenic plants that are individually larger than the wild-type plant in vegetative and/or reproductive structure. It is known in the art that certain growth factors may be used to increase plant and/or plant flower size. Unfortunately, application of such growth factors is costly and time consuming, and do not necessarily substantially increase the yield of seeds from the plants. A need, therefore, exists for developing an improved method for increasing the yield of seeds from plants.

SUMMARY OF THE INVENTION

[0007] In a first embodiment, the present invention provides a transgenic plant resistant to the effects of externally imposed stresses, wherein the transgenic plant comprises a nucleotide sequence comprising an exogenous tonoplast pyrophosphate driven H+ pump gene operably linked to a promoter. The transgenic plant of the invention is resistant to externally imposed stresses such as drought, prolonged exposure to temperatures below 0° C, and growth media high in salt content, where the growth media may be soil or water. Preferably, the exogenous tonoplast driven H+

pump gene is operably linked to a double tandem enhancer of the 35S CaMV promoter. In addition, the present invention contemplates a seed produced by the transgenic plant of the invention, as well as a progeny plant from the seed of the plant of the invention.

[0008] In another embodiment, the present invention provides a transgenic plant containing a polynucleotide sequence comprising a multiplicity of exogenous tonoplast pyrophosphate driven H+ pump genes operably linked to a double tandem enhancer of the 35S CaMV promoter, wherein the number of pyrophosphate driven H+ pump genes is sufficient to express a sufficient number of pyrophosphate driven H+ pumps on the vacuolar membranes to impart desirable phenotypic traits to the transgenic plant. Among these phenotypic traits are an ability to resist the effects of externally imposed stresses, wherein the externally imposed stresses to which the plant is resistant are exemplified by drought, prolonged exposure to temperatures below 0° C, and a growth medium high in salt content. The plant of this embodiment of the invention comprises exogenous nucleic acid that encodes AVP1 or, alternatively, an homolog of AVP1. This homolog may be obtained from tobacco, bacteria, tomato or corn. The transgenic plants of this embodiment comprise an *AVP1* gene in a construct designed to overexpress AVP1 or designed to down-regulate endogenous pyrophosphatase. In this construct, preferably the *AVP1* is operably linked to a double tandem enhancer of a 35S CaMV promoter. Preferably, the *AVP1* gene is derived from a wild type plant of the same species from which the transgenic plant is derived. Alternatively, the *AVP1* gene is derived from a wild type plant of a different species from which the transgenic plant is derived.

[0009] In another aspect of this embodiment of the present invention, the transgenic plant is larger than a wild-type plant of the same species. Also, the invention contemplates a progeny plant of the transgenic plant, seeds produced by the transgenic plant, and a progeny plant grown from the seed.

[0010] In yet another embodiment of the invention, the present invention contemplates a transgenic plant obtained by introducing into the genome of the plant exogenous nucleic acid that alters expression of vacuolar pyrophosphatase in the transgenic plant, as well as plant cells comprising exogenous nucleic acid that alters expression of vacuolar pyrophosphatase in the plant cell. Preferably, the plant cells are selected from the group consisting of root cells and stem cells. Furthermore, the plant cells comprise exogenous nucleic that acid encodes the AVP1 protein. Preferably, in the plant cells of the present invention, the exogenous nucleic acid that encodes AVP1 is

present in a construct designed to overexpress AVP1 or designed to down-regulate endogenous pyrophosphatase. More preferably, the construct comprises the *AVP1* gene operably linked to a chimeric promoter designed to overexpress AVP1. More preferably still, the *AVP1* gene is operably linked to a double tandem enhancer of a 35S CaMV promoter. In this embodiment, the nucleotide encoding AVP1 is derived from a wild type plant of the same species from which the transgenic plant is derived, although the present invention also contemplates that the nucleotide encoding AVP1 can be derived from a wild type plant of a different species from which the transgenic plant is derived.

[0011] The present invention, in yet another embodiment, provides a method of making a transgenic plant that is larger in size than its corresponding wild type plant comprising introducing into one or more cells of a plant a nucleotide sequence that alters expression of vacuolar pyrophosphatase in the plant to yield transformed cells, thereby producing a transgenic plant that is larger than its corresponding wild type plant. In this embodiment, the method further comprises regenerating plants from the transformed cells to yield transgenic plants and selecting a transgenic plant that is larger than its corresponding wild type plant, thereby producing a transgenic plant which is larger than its corresponding wild type plant. Also encompassed by this embodiment of the present invention is a transgenic plant produced by this method.

[0012] In yet another alternative embodiment, the present invention contemplates a method of increasing the yield of a plant comprising introducing into one or more cells of a plant nucleic acid that alters expression of vacuolar pyrophosphatase in the plant to yield transformed cells, thereby increasing the yield of the plant. this method further comprises regenerating plants from the transformed cells to yield transgenic plants and selecting a transgenic plant that is larger than its corresponding wild type plant, thereby increasing the yield of the plant.

[0013] Also provided by the present invention is a method of making a transgenic plant having increased flower size compared to its corresponding wild type plant comprising introducing into one or more cells of a plant nucleic acid that alters expression of vacuolar pyrophosphatase in the plant to yield transformed cells, thereby producing a transgenic plant having increased flower size compared to its corresponding wild type plant. preferably, the exogenous nucleic acid encodes AVP1. This embodiment of the invention also enompasses a transgenic plant produced by the method.

[0014] In another embodiment, the invention of the instant application provided a method of making a transgenic plant with increased biomass comprising introducing into one or more cells of a plant a nucleic acid construct that alters expression of vacuolar pyrophosphatase so as to increase vacuolar pyrophosphatase activity in the cell to yield transformed cells thereby producing a transgenic plant with increased biomass. This method further comprises regenerating plants from the transformed cells to yield transgenic plants and selecting a transgenic plant with increased biomass. Also encompassed by this embodiment of the present invention is a transgenic plant produced by the method.

[0015] In an alternate aspect of this embodiment, the present invention provides a method of making a transgenic plant with an increased biomass over its corresponding wild type plant, wherein the increased biomass relates to an increase in the biomass of a plant part selected from the group consisting of leaves, stems, roots, seeds, flowers, and fruits; said method comprising introducing into one or more cells of a plant an exogenous nucleic acid that alters expression of vacuolar pyrophosphatase such so as to enhance the activity of the vacuolar pyrophosphatase in the plant to yield transformed cells, thereby producing a transgenic plant with an increased biomass. This method, as provided by the present invention, further comprises regenerating plants from the transformed cells to yield transgenic plants and selecting a transgenic plant that is larger than its corresponding wild type plant, thereby producing a transgenic plant with an increased biomass. Preferably, according to this method, the exogenous nucleic acid encodes AVP1, or a homolog thereof. More preferably, the AVP1, or homolog thereof, is expressed from a construct designed to overexpress AVP1, or the homolog thereof. Preferably, this construct comprises the *AVP1* gene, or gene encoding a homolog of AVP1, wherein the gene is operably linked to a chimeric promoter designed to overexpress AVP1. More preferably, the *AVP1* gene is operably linked to a chimeric promoter selected from the group consisting of tissue specific promoters, constitutive promoters, inducible promoters and combinations thereof. In a most preferred embodiment, the *AVP1* gene is operably linked to a tissue-specific promoter that promotes expression of AVP1 in pollen.

[0016] In one aspect of this embodiment, the AVP1 gene is operably linked to a double tandem enhancer of a 35S CaMV promoter. Preferably, the *AVP1* gene, or homolog thereof, is derived from a wild type plant. Alternatively, the *AVP1* gene, or homolog thereof, is derived from a transgenic plant. In yet another aspect of this

embodiment, the *AVP1* gene, or homolog thereof, is derived from a mutant plant. in one aspect, the transgenic plant is grown in soil, or is grown hydroponically. Also possible, is that a cell from the transgenic plant is grown in culture. Also contemplated by this embodiment of the present invention is a transgenic plant produced by this method.

[0017] In still another embodiment, the present invention provides a method of making a transgenic plant having increased root structure compared to its corresponding wild type plant comprising introducing into one or more cells of the plant an exogenous nucleic acid that alters expression of vacuolar pyrophosphatase so as to increase vacuolar pyrophosphatase activity in the plant to yield transformed cells, thereby producing a transgenic plant having increased root structure. According to this embodiment of the present invention, the exogenous nucleic acid encodes AVP1, or a homolog thereof. Also contemplated here is a transgenic plant produced by the method.

[0018] In a most preferred embodiment, the present invention contemplates a method for increasing production of seeds in plants comprising the steps of (a) providing pollen from a first plant, wherein said first plant has been transformed with a tonoplast pyrophosphate driven H+ pump gene operably linked to a promoter to create a transgenic plant; (b) fertilizing a second plant of the same species from which the first plant is derived with the pollen from the transgenic plant; and (c) culturing the fertilized plant until the plant produces mature seeds. According to this method, the tonoplast pyrophosphatase driven H+ pump gene transformed into the first plant is exogenous. Also contemplated is that the second plant is a transgenic plant or a wild type plant.

[0019] Preferably, the exogenous tonoplast pyrophosphate driven H+ pump gene is operably linked to a chimeric promoter. More preferably, the exogenous tonoplast pyrophosphate driven H+ pump gene is operably linked to a double tandem enhancer of the 35S CaMV promoter. More preferably still, the exogenous tonoplast pyrophosphate driven H+ pump gene is operably linked to a double tandem enhancer of the 35S CaMV promoter and is further operably linked to a multiple cloning site. in a most preferred embodiment, the exogenous tonoplast pyrophosphate driven H+ pump gene encodes AVP1. Also coming within this embodiment of the present invention is a plant seed produced by the method; a progeny plant grown from the plant seed, wherein the first and second plants used in the method are from the species *A. thaliana*. Alternatively, the first and second plants are from the species *nicotinia tabacum*.

[0020] In another aspect of this embodiment of the invention, the second plant has been transformed with a polynucleotide sequence comprising an exogenous tonoplast pyrophosphatase driven H+ pump gene operably linked to a promoter. Preferably, the polynucleotide sequence comprises an exogenous tonoplast pyrophosphatase driven H+ pump gene operably linked to a double tandem enhancer of the 35S CaMV promoter. Further, the polynucleotide sequence comprises an exogenous tonoplast pyrophosphatase driven H+ pump gene operably linked to a double tandem enhancer of the 35S CaMV promoter and further operably linked to a multiple cloning site. More preferably, the polynucleotide sequence comprises an exogenous tonoplast pyrophosphatase driven H+ pump gene operably linked to a double tandem enhancer of the 35S CaMV promoter and further operably linked to a heterologous coding sequence. This aspect of the present embodiment also contemplates a plant seed produced by the method and a progeny plant grown from the plant seed.

[0021] The present invention provides, in one aspect, pollen produced by a transgenic plant that has been transformed with a tonoplast pyrophosphatase driven H+ pump gene operably linked to a promoter. The pollen from these transgenic plants is more competent in fertilization, resulting in increased yield of seeds from the plants. Because most crops of interest are hermaphroditic, self-pollination of the transgenic plants by the more competent pollen will occur and will result in improved seed yield. The improved seed yield is demonstrated both by increased numbers of seeds and increased seed pod mass. Increased seed yield can increase the production of products from cultivars such as rice, canola, wheat, corn and sunflower. Also, increased seed yield can decrease the cost of producing seeds to be used in crop production.

[0022] Other advantages of the present invention will become more readily apparent in view of the accompanying detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023] So that those having ordinary skill in the art to which the subject invention appertains will more readily understand the subject invention, reference may be had to the drawings, wherein:

[0024] *Fig. 1A* is an overhead view of wild type (WT) and two independent transgenic lines (AVP1-1 and AVP1-2) after 10 days of water deprivation.

[0025] *Fig. 1B* is an overhead view of the plants shown in Fig. 1A after rewatering.

[0026] *Fig. 2* is an overhead view of a representative wild type plant (WT) versus representative transgenic plants overexpressing AVP1 (AVP1-1 and AVP1-2) after exposure to 7 days of water deficit stress.

[0027] *Figs. 3A, 3B and 3C* are photomicrographs of the root and root hairs of representative five day old seedlings obtained from representative WT, AVP1-1 and AVP1-2 plants grown parallel to the surface on vertical plant nutrient agar plates.

[0028] *Fig. 4* is an immunoblot of membrane fractions isolated from wild type (WT) and two independent transgenic lines (AVP1-1 and AVP1-2) overexpressing AVP-1.

[0029] *Fig. 5* is a perspective view of wild-type plants (WT) versus representative transgenic plants overexpressing AVP-1 (AVP1-1 and AVP1-2) grown in salty soil.

[0030] *Fig. 6A* is a graph showing accumulation of sodium ion in leaf tissue for wild-type plants (WT) versus representative transgenic plants overexpressing AVP-1 (AVP1-1 and AVP1-2).

[0031] *Fig. 6B* is a graph showing accumulation of potassium ion in leaf tissue for wild-type plants (WT) versus representative transgenic plants overexpressing AVP-1 (AVP1-1 and AVP1-2).

[0032] *Fig. 6C* is a graph showing the results of measurements of transport using vacuolar membrane vesicles derived from the AVP1 transgenic plant demonstrating that vacuoles from these plants have enhanced cation uptake capability.

[0033] *Fig. 7 is a graph of the uptake of calcium into the 35SAVP-1 transgenic* vacuolar membrane vesicles (squares) of AVP1-2 *Fig. 5* versus calcium uptake into vesicles obtained from wild type (WT) of *Fig. 5*.

[0034] *Figs. 8A and 8B* are illustrations demonstrating the theorized mechanism for a higher accumulation of solids into vacuoles via a proton driven function versus that of WT vacuoles.

[0035] *Fig. 9* is a graph showing seed yield for wild-type, AVP1-1 and AVP1-2 plants grown in a 16 hour light / 8 hour dark cycle for two months.

[0036] *Fig. 10A* is a graph showing average number of seeds.

[0037] *Fig. 10B* is a graph showing average seed pod mass.

[0038] *Fig. 11* is a graph showing volume occupied by seeds from wild type and transgenic tobacco.

[0039] *Fig. 12A.* is a photomicrograph (40x) showing formation of pollen tubes in the papillae of the stigma of a wild type Arabidopsis thaliana plant pollinated with wild type pollen.

[0040] *Fig. 12B.* is a photomicrograph (40x) showing formation of pollen tubes in the papillae of the stigma of a wild type Arabidopsis thaliana plant pollinated with 35S AVP1 transgenic pollen.

[0041] *Fig. 13A.* Photomicrograph (40x) showing fluorescence of wild type tube forming pollen in wild type Arabidopsis thaliana plant six hours after application, stained using theaniline blue.

[0042] *Fig. 13B.* Photomicrograph (40x) showing fluorescence of 35S AVP1 tube forming pollen in wild type Arabidopsis thaliana plant six hours after application, stained using theaniline blue.

DETAILED DESCRIPTION OF THE INVENTION

[0043] Preferred embodiments of the invention are described below. The preferred embodiments disclosed herein are to be considered exemplary of the principles of the present invention and are not intended to limit the invention to the embodiments described. Various modifications will be apparent to those skilled in the art based on the teachings herein without departing from the scope or spirit of the invention disclosed herein.

[0044] Transgenic plants that overexpress a vacuolar proton-pumping pyrophosphatase have been shown to have improved resistance to stress, such as drought, high salinity or extended exposure to freezing conditions, and to exhibit enhanced meristematic activity. These transgenic plants, and methods for producing these plants, have been described in application Serial No. PCT/US00/30955, filed on November 10, 2000, and in application Serial No. PCT/US01/09548, filed on March 24, 2001, the entire contents of each of which are hereby incorporated by reference.

[0045] Briefly, this transformation can be accomplished using, for example, an exogenous tonoplast pyrophosphate driven H+ pump gene operably linked to a

promoter. The exogenous gene may encode AVP-1 or a homologue of AVP-1. The invention is not limited in this regard, and pollen from any plant that has been transformed to overexpress a vacuolar proton-pumping pyrophosphatase may be used.

[0046] The promoter may be a chimeric promoter, a double tandem enhancer of the 355 CaMV promoter, a pollen specific promoter, or any other promoter known to those of skill in the art. Alternatively, the plant may be transformed using a polynucleotide sequence comprising an exogenous tonoplast pyrophosphatase drive H+ pump gene operably linked to a promoter.

[0047] The present invention discloses a transgenic plant having upregulated expression of vacuolar pyrophosphatase. It has been found that plants displaying such upregulated activity are, in general larger than wild-type counterparts, demonstrate improved stress resistance to drought and/or freeze, and have increased tolerance to salt in the media in which they are growing.

[0048] Any suitable exogenous nucleic acid molecule which alters expression of vacuolar pyrophosphatase in the plant can be used to transform the transgenic plants in accord with the present invention. The exogenous nucleic acid can comprise nucleic acid that encodes a vacuolar pyrophosphatase protein (an exogenous vacuolar pyrophosphatase), such as AVP1, a functional portion thereof (peptide, polypeptide), or a homologue thereof, and/or nucleic acid that alters expression of the endogenous vacuolar pyrophosphatase of the plant into which the exogenous nucleic acid is introduced. By "exogenous nucleic acid" it is meant a nucleic acid from a source other than the plant cell into which it is introduced, or into a plant or plant part from which the transgenic part was produced. The exogenous nucleic acid used for transformation can be RNA or DNA, (*e.g.,* cDNA, genomic DNA). In addition, the exogenous nucleic acid can be circular or linear, double-stranded or single-stranded molecules. Single-stranded nucleic acid can be the sense strand or the anti-sense strand. By a "functional portion" of a nucleic acid that encodes a vacuolar pyrophosphatase protein it is meant a portion of the nucleic acid that encodes a protein or polypeptide which retains a functional characteristic of a vacuolar pyrophosphatase protein. In a particular embodiment, the nucleic acid encodes AVP1, a functional portion or a homologue thereof.

[0049] Nucleic acid that alters expression of the endogenous vacuolar pyrophosphatase of the plant into which the exogenous nucleic acid is introduced includes regulatory sequences (*e.g., inducible, constitutive*) which function in plants and

antisense nucleic acid. Examples of regulatory sequences include promoters, enhancers and/or suppressors of vacuolar pyrophosphatase. The nucleic acid can also include, for example, polyadenylation site, reporter gene and/or intron sequences and the like whose presence may not be necessary for function or expression of the nucleic acid but can provide improved expression and/or function of the nucleic acid by affecting, for example, transcription and/or stability (*e.g.*, of mRNA). Such elements can be included in the nucleic acid molecule to obtain optimal performance of the nucleic acid.

[0050] The nucleic acid for use in the present invention can be obtained from a variety of sources using known methods. For example, the nucleic acid encoding a vacuolar pyrophosphatase (*e.g.*, AVP1) for use in the present invention can be derived from a natural source, such as tobacco, bacteria, tomato or corn. In one embodiment, the nucleic acid encodes a vacuolar pyrophosphatase that corresponds to a wild type of the transgenic plant. In another embodiment, the nucleic acid encodes a vacuolar pyrophosphatase that does not correspond to a wild type of the transgenic plant. Nucleic acid that alters expression of the endogenous vacuolar pyrophosphatase of the plant into which the exogenous nucleic acid is introduced (*e.g.*, regulatory sequence) can also be chemically synthesized, recombinantly produced and/or obtained from commercial sources.

[0051] A variety of methods for introducing the nucleic acid of the present invention into plants are known to those of skill in the art. For example, Agrobacterium-mediated plant transformation, particle bombardment, microparticle bombardment (*e.g.*, U.S. Patent No. 4,945,050; U.S. Patent No. 5,100,792) protoplast transformation, gene transfer into pollen, injection into reproductive organs and injection into immature embryos can be used. The exogenous nucleic acid can be introduced into any suitable cell(s) of the plant, such a root cell(s), stem cell(s) and/or leaf cell(s) of the plant. In addition, the genetic material of the transformed plant may be introduced into other germ lines displaying traits of interest by cross-breeding the transgenic lines with other lines in accord with established principles of Mendelian genetics.

[0052] Any suitable plant can be used to produce the transgenic plants of the present invention. For example, tomato, corn, tobacco, rice, sorghum, cucumber, lettuce, turf grass, ornamental (*e.g.*, larger flowers, larger leaves) and legume plants can be transformed as described herein to produce the transgenic plants of the present invention. In addition, the transgenic plants of the present invention can be grown in any medium which supports plant growth such as soil or water (hydroponically).

[0053] Desirable phenotypic traits (*e.g.*, the ability to resist externally imposed stresses, including conditions of decreased water supply or extended periods of below-freezing temperatures) may be introduced into a plant of the present invention by transforming plant cells with exogenous nucleic acid which alters the expression of vacuolar pyrophosphatase in the plant such that expression is upregulated. Any suitable vacuolar pyrophosphatase, several of which have been cloned, can be used in the compositions and methods of the present invention (*e.g.*, Sarasian, Z., *et al., Proc. Natl. Acad. Sci., USA, 89:*1775-1779 (1992); Jenslerchl, *et al., Molec. Biol., 29:* 833-840 (1995); Kim, Y., *et al., Plant Physiol., 106:*375-382 (1994)). In a particular embodiment, the present invention relates to a transgenic plant exhibiting desirable phenotypic traits such as resistance to external stresses comprising an exogenous nucleic acid construct which is designed to overexpress AVP1 (Sarasian, Z., *et al., Proc. Natl. Acad. Sci., USA, 89:*1775-1779 (1992)). Transformation of the plant cells may be carried out in a whole plant, seeds, leaves, roots or any other plant part. Such transgenic plants are preferably altered such that they grow in a concentration of salt that inhibits growth of a corresponding non-transgenic plant. Transgenic progeny of the transgenic plants, seeds produced by the transgenic plant and progeny transgenic plants grown from the transgenic seed, which are also the subject of the present invention, advantageously carry such salt tolerant trait. Plants may be regenerated from transformed cells to yield transgenic plants, which may be screened for certain levels of salt tolerance. In a preferred embodiment, the exogenous nucleic acid encodes AVP1, or a homologue thereof.

[0054] Drought and/or freeze tolerance may be introduced into plants by transforming plant cells with exogenous nucleic acid which alters the expression of vacuolar pyrophosphatase in the plant such that such expression is upregulated. In a preferred embodiment there is provided a substantially drought and/or freeze resistant transgenic plant which comprises a genome having one or more exogenously introduced vacuolar H^+-translocating pump genes. A particularly preferred fertile transgenic plant eliciting drought and/or freeze tolerance, as well as the ability to grow in saline soils, comprises an isolated exogenous chimeric DNA construct encoding vacuolar H^+-translocating pump, preferably operably linked to a promoter, such as the 35S CaMV promoter or any other promoter, including, without limitation, tissue specific promoters. The transgenic plant may contain a polynucleotide sequence comprising an exogenous tonoplast pyrophosphate H^+ pump gene operably linked to a promoter. In yet another particularly preferred drought and/or freeze resistant transgenic plant having the capacity

to grow in saline soils, the polynucleotide sequence comprises an exogenous tonoplast pyrophosphate H^+ pump gene operably linked to a double tandem enhancer of the 35S promoter. A particularly preferred tonoplast pyrophosphate H^+ pump gene is the AVP1 gene.

[0055] Previous work has shown that a decrease in the levels of the A subunit of the vacuolar H^+-ATPase of carrot, using an antisense construct, resulted in a plant with reduced cell expansion and altered leaf morphology (J. P. Gogarten, et al., *The Plant Cell* 4, 851-864 (1992)). The present inventor has hypothesized that an increased supply of H^+ into the vacuole could cause cell expansion. Recently, based on the theory that as the availability of protons in the vacuolar function of ion accumulation, it has been hypothesized by the same inventor that accumulation of solids in the vacuoles might be useful to protect against draught and to provide for a more freeze resistant plants.

[0056] Since plant vacuoles constitute 40 to 99% of the total intracellular volume of a mature plant cell, changes in the size of the vacuole have dramatic effects upon cell size (R. G. Zhen, E. J. Kim, P. A. Rea, in *The Plant Vacuole* . (Academic Press Limited, 1997), vol. 25, pp. 298-337). The volume of the vacuole is controlled by ion and water fluxes mediated by pumps and transporters. In plants the driving force that triggers the movement of ions, solutes and water across membranes is a proton gradient. The activity of the vacuolar H^+-pumps results in luminal acidification and the establishment of a H^+ electrochemical potential gradient across the vacuolar membrane, which powers the secondary active transporters of inorganic ions, sugars, and organic acids. The activity of these transporters modulates cellular pH and ion homeostasis and leads to the accumulation of solutes required to generate the osmotic potential that promotes vacuolar expansion (H. Sze, X. Li, M. G. Palmgren, *The Plant Cell* 11, 677-689 (1999)).

[0057] There are three distinct pumps that generate proton electrochemical gradients. One at the plasma membrane that extrudes H^+ from the cell (PM H^+-ATPase) and two at the vacuolar membrane or other endomembrane compartments that acidify their lumen (the vacuolar type H^+-ATPase and H^+-PPase) (R. A. Leigh, in *The Plant Vacuole* L. a. Sanders, Ed. (Academic Press, San Diego, California, 1997), vol. 25, pp. 171-194.).

[0058] The present inventor has recognized that plants have a number of vacuolar H^+-translocating pumps, and that by upregulating their activity, increasing their expression, upregulating their transcription and/or translation, or increasing their copy number that one can increase accumulation of solids in the vacuole due to an increase in

the availability of protons in the vacuoles. The inventor tested this hypothesis by increasing the copy number of the vacuolar H^+-translocating pump, the inorganic pyrophosphatase or V-PPase that consists of a single polypeptide (R. G. Zhen, E. J. Kim, P. A. Rea, in *The Plant Vacuole* . (Academic Press Limited, 1997), vol. 25, pp. 298-337). In *Arabidopsis* the V-PPase encoded by the *AVP-1* gene is capable of generating a H^+ gradient across the vacuole membrane (tonoplast) similar in magnitude to that of the vacuolar H^+-ATPase (V. Sarafian, Y. Kim, R. J. Poole, P. A. Rea, *Proc. Natl. Acad. Sci.* 89, 1775-1779 (1992)). As would be understood by one of ordinary skill in the art, similar genes in other plants should function in a similar manner.

[0059] It is known that H^+-PPase is the main proton pump of vacuolar membranes in growing tissue. The later may be due to the fact that in growing tissue, nucleic acids, DNA, RNAs, proteins and cellulose etc. are actively being synthesized for the construct of the new cells, and as a result, a large amount of PP is produced as a by-product of these metabolic processes. The energy stored in the PP molecule may be transformed into a different source of energy, namely a H^+-gradient across the vacuolar membrane. This H^+-gradient constitutes the driving force for the vacuolar accumulation of solutes that generate the sufficient osmotic differential that enables the plant cell to initiate growth. While the present invention is not limited in any manner to any particular hypothesis for the increased growth effects seen, the present inventor has hypothesized that in transgenic plants overexpressing AVP-1 that the greater number of H^+-PPases has a positive effect on the velocity of the generation of the H^+ gradient, rendering a more active meristem.

[0060] To demonstrate the effect that an increased supply of H^+ into the vacuole would have on resistance to drought and/or freeze, and tolerance to salt growth, as well as size of the plants, the present inventor generated transgenic plants containing extra copies of a vacuolar proton pump, *AVP-1*.

[0061] *Arabidopsis thaliana* plants were transformed with constructs containing the *AVP-1* gene. Transgenic lines containing extra copies of this gene were then isolated. The *AVP-1*, open reading frame was cloned into the Xma1 site of a modified pRT103 [R. Topfer, V. Matzeit, B. Gronenborn, J. Schell and H-H. Steinbiss, *Nucleic acid Research* 15, 5890 (1987)]. This vector contains a tandem repeat of the 35-S promoter. A HindIII fragment containing the 35-S tandem promoter, AVP-1 ORF and the polyadenylation signal was subcloned into the HindIII site of the pPZP212 vector [P. Hajdukiewicz, Z. Svab and P. Maliga, *Plant Molecular Biology* 25, 989-994 (1994)].

Agrobacterium-mediated transformation was performed via vacuum infiltration of flowering *Arabidopsis thaliana (ecotype columbia).* Transgenic plants were selected by plating seeds of the transformed plants on plant nutrient agar plates supplemented with 25 mg/liter kanamycin. Plants were subsequently selected for two generations to identify transgenic plant homozygous for the transgene.

[0062] Wild type and AVP1 transgenic plants were tested for drought tolerance by growing the plants under conditions of water deprivation. Plants were grown for four weeks under fully watered regimen at 21° C (standard culture conditions) and then transferred into a chamber with a 3° C warmer temperature and no further addition of water. After 10 days of water deprivation (Fig. 1A), plants from each group were rewatered (Fig. 1B). Deprivation of water for this time was lethal to wild type plants, but plants from both transgenic lines, *AVP1-1* and *AVP1-2*, survived and continued normal growth, bolted, and set viable seeds.

[0063] *Fig. 2* is an overhead view of a representative wild type plant (WT) versus representative transgenic plants overexpressing AVP1 (AVP1-1 and AVP1-2) after exposure to 7 days of water deficit stress. Wild type and transgenic plants overexpressing *AVP-1* were tested for drought tolerance (24°C). After 7 days of water deficit stress wild type (WT) plants withered, whereas plants from both *35S AVP-1* transgenic lines (AVP1-1 and AVP1-2) were turgid and alive. Furthermore, when the drought stressed plants were then watered, transgenic plants pursued normal growth, bolted and set seeds, whereas wild type plants died. The relative water content of leaves from wild type and *35SAVP-1* transgenic plants were determined along the water deficit stress,. demonstrating increased water retention by the transgenic lines as compared to the WT plants.

[0064] While not illustrated in the accompanying illustrations, similar results may be seen with respect to freeze challenge (< 0° C) over a 24-hour or greater period for a number of plant species. While not limited to such hypothesis, transgenic plants overexpressing AVP1 (AVP1-1 and AVP1-2) are believed to provide enhanced protection from freeze as compared to wild type (WT) plants due to the higher amounts of cations in the vacuoles. Higher amounts of cations confer a greater osmotic pressure that leads to a greater water retention capability endowing the plants not only with the ability to withstand low soil water potentials, but also providing greater protection from freezing that leads to significant desiccation of the plants.

[0065] *Fig. 3A, Fig. 3B and Fig. 3C are* photomicrographs (magnification: times 40; bar length on photograph = 2mm) of the root and root hairs of representative five day old seedlings obtained from representative WT, AVP1-1 transgenic and AVP1-2 transgenic of *Fig. 3A* grown parallel to the surface on vertical plant nutrient agar plates. Seedlings of both transgenic lines AVP1-1 and AVP1-2 showed root hairs with an average length 40 and 70% larger than wild-type (WT) root hairs (Root hair length along the whole root was determined from five members of each set of seedlings. An average of 80 root hairs per plant were measured). The length of the root hairs is correlated with the size of the vacuole, so the increased size of the root hair is likely to result from increased vacuolar volume. This compares with the *Arabidopsis* mutant *rdh3* which has been reported to have reduced vacuolar volume and is a short plant with abnormally short root hairs (M. E. Galway, J. W. J. Heckman, J. W. Schiefelbein, *Planta* **201**, 209-218 (1997)). A well characterized mode of plant cell expansion is tip growth, whereby new cell growth is limited to a single growing point and leads to the formation of a tubular-shaped cell. *Plant Physiol.* 103: 979-985 (1993). Two cell tips that are known to display this pattern of tip growth are pollen tubes and root hairs. For root hairs, it is expected that the increased root structure will have a positive impact on soil erosion, nitrogen fixation in legumes, and will aid in water and nutrient uptake by the plant.

[0066] *Fig. 4* is an immunoblot of membrane fractions isolated from wild type (WT) and two independent transgenic lines (AVP1-1 and AVP1-2) overexpressing AVP1. Total membrane fractions were isolated from shoots of eight week old wild type (WT) and AVP-1 transgenic plants (AVP1-1 and AVP1-2) grown in a hydroponic media for 6 weeks. Shoots of plants homogenates were sequentially centrifuged for 15 and 30 min at 8 and 100 kg respectively. The 100kg membrane pellet was re-suspend in 10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol and 1 mM PMSF Protein (10 mg) was separated on a 10% SDS-PAGE, electroblotted and immunostained with antibodies raised against a KLH-conjugated synthetic peptide corresponding to the putative hydrophilic loop IV of the AVP-1 protein (V. Sarafian, Y. Kim, R. J. Poole, P. A. Rea, *Proc. Natl. Acad. Sci.* **89**, 1775-1779 (1992)). PPase was detected by chemiluminescence. *Fig. 1C* illustrates that the transgenic lines (1' and 2') express AVP-1 protein at higher levels than the wild type (WT) (that is, 1' = 15% and 2' =50% more than WT).

[0067] As wheat that has been deprived of water is rendered more drought tolerant by an increase in cell K^+ content (from 100 mM to 300 mM) (S. Gupta, G.

Berkowitz, P. Pier, *Plant Physiol* **89**, 1358-1365 (1989)), it is hypothesized (but the invention is not hereby limited by such theory) that the increased drought resistance of the AVP-1 transgenic plants may be a consequence of their higher vacuolar concentration of potassium that results in a increased water retention capability. Laboratory tests appear to confirm this.

[0068] *Fig. 5* is a perspective view of wild type plants (WT) versus representative transgenic plants overexpressing AVP1 (AVP1-1 and AVP1-2) grown in salty soil. Five wild-type plants (WT) and two AVP-1 overexpressing transgenic lines (AVP1-1 and AVP1-2) were grown on soil in a 10 hour light/dark cycle. Plants were watered with a diluted nutrient solution (1/8 MS salts) for six weeks and subsequently watered with a diluted nutrient solution supplemented with NaCl. The concentration of NaCl began with 100 mM and was increased every four days by 100 mM. The photograph in *Fig. 3* corresponds to plants at the tenth day in the presence of 300 mM NaCl. *Fig. 3* illustrates that the two AVP-1 plant types (AVP1-1 and AVP1-2) were significantly hardier in salty soil as compared to wild-type plants. The fact that genetically engineered *Arabidopsis thaliana* plants that overexpress either *AVP1* (the pyrophosphate-energized vacuolar membrane proton pump, this work) or *AtNHX1* (the Na^+/H^+ antiporter, (Apse, M., *et al., Science, 285*:1256-1258 (1999)) and this work) are capable of growing in the presence of high NaCl concentrations strongly supports the strategy described herein. A double transgenic plant would be expected to demonstrate a further enhanced salt-tolerant phenotype. These *Arabidopsis thaliana* transporters or their counterparts may perform similar function in important agricultural crops.

[0069] **A Working Model of Cation Homeostasis in Plant Organelles** While the present invention is not limited to any particular hypothesis, the present inventors have developed a working model for cation homeostasis in plant cells which can explain the observed results with respect to the transgenic plants disclosed herein.

[0070] In plants, most of the transport processes are energized by the primary translocation of protons. H^+-translocating pumps located at the plasma membrane and tonoplast translocated H^+ from the cytosol to extracellular and vacuolar compartments, respectively (Rea, P.A., *et al.,* Tonoplast Adenosine Triphosphate and inorganic Pyrophosphatase. In: *Methods Plant Biochem.*, pp. 385-405, Academic Press Limited, London (1990)). The plant tonoplast contains two H^+-translocating pumps; the V-ATPase and the inorganic pyrophosphatase or V-PPase. Their action results in luminal

acidification and the establishment of a H^+ electrochemical potential gradient across the tonoplast (Davies, J.M., *et al.,* The Bioenergetics of Vacuolar H^+ Pumps. In: *Plant Vacuole*, pp. 340-363, Leigh, R.A., Sanders, D. (eds.), Academic Press, San Diego (1997)). The vacuolar membrane is implicated in a broad spectrum of physiological processes that include cytosolic pH stasis, compartmentation of regulatory Ca^{2+}, sequestration of toxic ions such as Na^+, turgor regulation, and nutrient storage and retrieval. The vacuole constitute 40 to 99% of the total intracellular volume of a mature plant cell. The vacuolar proton pumping pyrophosphatase is a universal and abundant component of plant tonoplast capable of generating a steady-state trans-tonoplast H^+ electrochemical potential similar or greater than the one generated by the V-ATPase (Rea, P.A., *et al.,* Tonoplast Adenosine Triphosphate and Inorganic Pyrophosphatase. In:*Methods Plant Biochem.*, pp. 385-405, Academic Press Limited, London (1990)). Pyrophosphate (PPi) is a by-product in the activation or polymerization steps of a wide range of biosynthetic pathways and in plants serves as an alternative energy donor to ATP for sucrose mobilization via sucrose synthetase, for glycolysis via PPi: fructose-6-phosphate phosphotransferase and for tonoplast energization via the vacuolar proton pumping pyrophosphatase (Stitt, M., *Bot. Acta 111*:167-175 (1998)).

[0071] Most of intracellular organelles, including clathrin-coated vesicles, endosomes, Golgi membranes and vacuoles have acidic interiors (Xie, X. S., *et al., J. Biol. Chem., 264*:18870-18873 (1989)). This acidification is mediated by a proton-translocating electrogenic ATPase and in plant vacuoles also via a pyrophosphate-driven proton pump V-PPase (Davies, J.M., *et al.,* The Bioenergetics of Vacuolar H^+ Pumps. In: *Leigh R.A., Sanders, D., (eds) The Plant Vacuole*, pp. 340-363, Academic Press, San Diego (1997); Zhen, R.G., *et al.,* "The Molecular and Biochemical Basis of Pyrophosphate-Energized Proton Translocation at the Vacuolar Membrane Academic Press Limited (1997)). There exists a requirement of anion transport to maintain net electroneutrality (al-Awqati, A., *Curr. Opin. Cell. Biol., 7*:504-508 (1995)).

[0072] Two transgenic lines of *Arabidopsis thaliana* were analyzed, *AVP1-1* and *AVP1-2*. Each line contains extra copies of the *35S::AVP1* gene inserted at a single chromosomal location. Analysis of AVP1 protein levels in membrane fractions isolated from shoots show that these transgenic plants express more AVP1 protein than does the wild type (*AVP1- 1*, 1.6 fold and *AVP1- 2*, 2.4 fold increase over wild type, P-value = 0.0005) (Fig. 1) as determined from four independent Western blots. The differences between these transgenic plants could be due to the number of copies of *AVP1* inserted

into the genome or the sites of insertion. The transgenic plants overexpressing AVP1 are more salt tolerant than wild type plants (Figs. 2 and 3). Plants from both *AVP1-1* and *AVP1-2* transgenic lines grow well in the presence of up to 250 mM NaCl whereas wild type plants grow poorly and exhibit chlorosis. After 10 days in these conditions wild type plants die, whereas the transgenic plants continue to grow well.

[0073] The enhanced tolerance to salinity and drought in transgenic plants with increased levels of AVP1 is most easily explained by an enhanced uptake of toxic cations such as sodium into the vacuole. Presumably, the greater AVP1 activity provides increased H^+ to drive the secondary active uptake of cations into the lumen of the vacuole (Fig. 2C). If so, there must be a compensatory transport of anions to maintain electroneutrality. The resulting elevated vacuolar solute content would confer greater water retention, permitting plants to survive under conditions of low soil water potentials. Furthermore, at high Na^+ concentrations, the increased H^+ gradient could also enhance the driving force for AtNHX-1-mediated Na^+/H^+ exchange, thereby contributing to the Na^+ sequestration into the vacuole of *AVP1* transgenic plants. Presumably, any toxic effects intrinsic to Na^+ are mitigated by this sequestration in the vacuole. This scenario predicts that a transgenic plant engineered to overexpress both, the AVP1 H^+-pump and the AtNHX1 Na^+/H^+ antiporter would tolerate even higher NaCl stresses than *AVP1* and *AtNHX1* single transgenic plants.

[0074] *Fig. 6* is a graph of Na^+ and K^+ content of wild-type plants (WT) versus representative transgenic plants overexpressing AVP-1 (1′ and 2′) grown in salty soil. Five wild-type plants (WT) and two AVP-1 overexpressing transgenic lines (1′ and 2′) were grown on soil in a 10 hour light/dark cycle. Plants were watered with a diluted nutrient solution (1/8 MS salts) for six weeks and subsequently watered with a diluted nutrient solution supplemented with NaCl. The concentration of NaCl began with 100 mM and was increased every four days by 100 mM. The photograph corresponds to plants at the tenth day in the presence of 300 mM NaCl. Parts of the plant above ground were harvested after 24 hours in the presence of 200 mM NaCl and their fresh weigh measured. After 48 hours at 75°C, the dry weight was measured. Na^+ and K^+ content was determined by atomic absorption. Values in the graphs of Fig. 4 are the mean +/- SE (n = 4). As can be seen from the graphs Na^+ and K^+ content in the transgenic lines (1′ and 2′) was significantly higher than that of wild-type counterparts.

[0075] *Fig. 7* is a graph of the uptake of calcium into the 35SAVP-1 transgenic vacuolar membrane vesicles (squares) of AVP1-2 versus calcium uptake into vesicles

obtained from wild type (WT). Wild-type plants (open circles) and transgenic plants from line AVP1-2 were grown hydroponically for nine weeks on a 10 hour light cycle. Vacuolar membrane vesicles were added to buffer containing 250 mM sorbitol, 25 mM BTP-Hepes pH 8.0, 50 mM KCl, 1.5 nM $MgSO_4$ and 10 μM Ca^{++}. This mix was incubated at 20°C for 10 minutes before adding 200 μM PPi to trigger the reaction. Ca^{++} ionophore A23187 was added to a final concentration of 5 μg/ml to dissipate the Ca^{++} gradient. Aliquot (200 μl) were filtered at the indicated times and washed with cold buffer as described (11). As is evidenced by the graphs, the transgenic plants from line 2' have greater calcium uptake than wild-type plants.

[0076] The above data is consistent with the hypothesis that transgenic plants overexpressing *AVP-1* have an enhanced H^+ pumping capability at their tonoplast and that an enhanced H^+ supply results in greater ion accumulation in the vacuole through the action of H^+-driven ion transporters. To further support this theory, Ca^{++} uptake capability of wild type and transgenic vacuolar membrane vesicles was determined.

[0077] It is well documented that Ca^{++} enters the plant vacuole via a Ca^{++}/H^+ antiporter (K. S. Schumaker, H. Sze, *Plant Physiol.* **79**, 1111-1117 (1985)). Furthermore, the genes encoding the *Arabidopsis thaliana* Ca^{++}/H^+ antiporters *CAX1* and *CAX2* have been isolated and characterized (K. D. Hirschi, R.-G. Zhen, K. W. Cunningham, P. A. Rea, G. R. Fink, *Proc. Natl. Acad. Sci. USA* **93**, 8782-8786 (1996)). *Fig. 8* shows that Ca^{++} uptake in the *35SAVP-1* transgenic vacuolar membrane vesicles is 36% higher than it is in vesicles obtained from wild type. Application of the Ca^{++} ionophore A23 lowered the $45Ca^{++}$ counts to background levels demonstrating the tightness of the vesicles (Fig 8) (K. S. Schumaker, H. Sze, *Plant Physiol.* **79**, 1111-1117 (1985)).

[0078] While not limited by such theory, a model consistent with the enhanced drought and freeze tolerance of the transgenic plants overexpressing the *AVP-1* gene is depicted in *Figs. 8A and 8B*. The model depicts how an increase in the number of AVP-1 pumps in the vacuole of transgenic plants can provide more H^+ that will permit the secondary transporters to import greater amounts of cations into the lumen of the vacuoles. Higher amounts of cations confer a greater osmotic pressure that leads to a greater water retention capability endowing plants to withstand low soil water potentials.

[0079] The present invention relates, in one aspect, to pollen produced by a transgenic plant transformed with a tonoplast pyrophosphatase driven H+ pump gene

operably linked to a promoter. In a second aspect, the invention relates to methods for increasing the production of seeds in plants using pollen from a transgenic plant transformed with a tonoplast pyrophosphatase driven H+ pump gene operably linked to a promoter.

[0080] Transgenic plants that overexpress a vacuolar proton-pumping pyrophosphatase, such as for example AVP 1, also produce an increased yield of seeds. Referring to Fig. 1, the seed yield, as expressed by the weight of seeds produced, is higher for AVP1-1 transgenic plants as compared to wild type plants. This increased seed yield is a result of the pollen from the transgenic plant having an enhanced ability to fertilize, referred to herein as fertilization competence.

[0081] To demonstrate that the improved seed yield is a result of the improved fertilization competence of the pollen from the transgenic plant, the pollen from wild type *Arabidosis thaliana* plants was used for pollination of two lines of transgenic *Arabidosis thaliana* plants transformed to overexpress AVP 1 (these two lines of transgenic plants are referred to herein as AVP 1-1 and AVP 1-2). Referring to Figs. 2A and 2B, the transgenic plants pollinated with pollen from wild type plants produced an average of between about 15 and 20 seeds, with an average seed pod mass of between about 2.5 and 3 milligrams. These results were compared to the seed yield obtained when pollen from the two lines of *Arabidosis thaliana* transgenic plants was used to pollinate wild type *Arabidosis thaliana* plants. Referring again to Figs. 2A and 2B, the wild type plants fertilized with transgenic pollen produced an average of between about 30 and 35 seeds, with an average seed pod mass of between about 4 and 5 milligrams.

[0082] These results demonstrate that pollen from transgenic plants transformed with a tonoplast pyrophosphatase-driven H^+ pump gene is capable of causing improved seed yield in plants fertilized with the transgenic pollen. To further illustrate, wild type plants that are fertilized with transgenic pollen also produce an increased yield of seeds, while transgenic plants fertilized with wild type pollen do not. These results clearly indicate that it is the pollen from the transgenic plant, and not the female reproductive organs of the transformed plant, that causes improved seed yield.

[0083] Similar results have also been observed in other plant species transformed to overexpress a vacuolar proton-pumping pyrophosphatase Referring to Fig. 3, the volume of seeds produced by wild type tobacco plants is compared to the seed pod volume produced in transgenic tobacco plants transformed to overexpress a vacuolar proton-pumping pyrophosphatase. The volume of five seed pods from each plant was

weighed. For the wild type tobacco plants, the volume of seeds in five pods was between about 0.5 milliliters and 0.8 milliliters. For the three lines of transgenic tobacco plants tested, the volume of seeds in five pods was between about 1.2 milliliters and 1.4 milliliters. The transgenic tobacco lines were crossed and the volume of five seed pods was measured. The volume of five seed pods from the crossed lines of tobacco plants remained between about 1.2 milliliters and 1.4 milliliters.

[0084] These results further demonstrate that it is the pollen from the transgenic plants that improve seed yield. All three lines of transgenic tobacco plants had substantially greater seed pod volume than wild type plants. In addition, when the three lines of transgenic tobacco plants were crossed, the seed pod volume was about the same as the seed pod volume of the uncrossed transgenic line, and much greater than the seed pod volume from the wild type line.

[0085] Most food plants of interest are hermaphroditic and will self-pollinate. Transgenic plants of this type that have been transformed to overexpress a vacuolar proton-pumping pyrophosphatase will themselves produce increased seed yields as a result of the improved fertilization competence of their pollen.

[0086] In another aspect of the present invention, which is especially useful for plant species which do not self-pollinate, pollen is provided from a transgenic plant transformed with a tonoplast pyrophosphatase driven H+ pump gene operably linked to a promoter. The pollen from the transgenic plant is used to fertilize transgenic or wild type female flowers. Fertilization of the wild type plant is accomplished using any appropriate method known to one skilled in the art.

[0087] After the wild type plant has been fertilized, the plant is cultured until the wild type plant produces mature seeds. The mature seeds are harvested from the wild type plant after they reach maturity. This increase in seed yield is a result of the improved competence of the pollen from the transgenic plant in fertilization.

[0088] In another embodiment of the present invention, the pollen from a transgenic plant transformed with a tonoplast pyrophosphatase driven H+ pump gene operably linked to a promoter is used to fertilize a transgenic plant which has also been transformed with a tonoplast pyrophosphatase driven H+ pump gene operably linked to a promoter. After the transgenic plant has been fertilized, the plant is cultured until it produces mature seeds. The mature seeds are harvested from the transgenic plant after they reach maturity.

[0089] As will be recognized by those of ordinary skill in the art based on the teachings herein, numerous changes and modifications may be made to the above-described embodiments of the present invention without departing from its scope or spirit. Accordingly, this detailed description of the invention is to be taken in an illustrative rather than a limiting sense.

I claim:

1. A transgenic plant resistant to the effects of externally imposed stresses, wherein the transgenic plant comprises a nucleotide sequence comprising an exogenous tonoplast pyrophosphate driven H+ pump gene operably linked to a promoter.

2. The transgenic plant of Claim 1, wherein the externally imposed stresses to which the plant is resistant are selected from the group consisting of drought, prolonged exposure to temperatures below O° C, and a growth medium high in salt content.

3. The transgenic plant of Claim 2, wherein the growth medium is selected form the group consisting of soil and water.

4. The transgenic plant of claim 1, wherein the exogenous tonoplast driven H+ pump gene encodes AVP1, or a homolog thereof.

5. The method of Claim 4, wherein the AVP1, or homolog thereof, is encoded by a gene present in a construct designed to overexpress AVP1, or homolog thereof.

6. The method of Claim 4, wherein the construct comprises the *AVP1* gene, or homologue thereof, operably linked to a chimeric promoter designed to overexpress AVP1.

7. The method of Claim 4, wherein the *AVP1* gene or homologue thereof is operably linked to a chimeric promoter selected from the group consisting of tissue specific promoters, constitutive promoters, inducible promoters and combinations thereof.

8. The method of Claim 4, wherein the *AVP1* gene is operably linked to a tissue-specific promoter that promotes expression of AVP1 in pollen.

9. The method of Claim 4, wherein the *AVP1* gene, or homolog thereof, is operably linked to a double tandem enhancer of a 35S CaMV promoter.

10. The method of Claim 4, wherein the *AVP1* gene, or homolog thereof, is derived from a wild type plant.

11. The method of Claim 4, wherein the AVP1, or homolog thereof, is derived from a transgenic plant.

12. A seed produced by the transgenic plant of Claim 1.

13. A progeny plant from the seed of Claim 12.

14. A transgenic plant obtained by introducing into the genome of the plant exogenous nucleic acid that alters expression of vacuolar pyrophosphatase in the transgenic plant.

15. Plant cells comprising exogenous nucleic acid that alters expression of vacuolar pyrophosphatase in the plant cell.

16. The plant cells of Claim 15, wherein the cells are selected from the group consisting of root cells and stem cells.

17. The plant cells of Claim 15, wherein the exogenous nucleic acid encodes AVP1.

18. The plant cells of Claim 17, wherein the AVP1 is derived from a wild type plant of the same species from which the transgenic plant is derived.

19. The plant cells of Claim 17, wherein the AVP1 is derived from a wild type plant of a different species from which the transgenic plant is derived.

20. A method for increasing production of seeds in plants comprising the steps of:

(a) providing pollen from a first plant, wherein said first plant has been transformed with a tonoplast pyrophosphate driven H+ pump gene operably linked to a promoter to create a transgenic plant;

(b) fertilizing a second plant of the same species from which the first plant is derived with the pollen from the transgenic plant; and

(c) culturing the fertilized plant until the plant produces mature seeds.

21. The method of Claim 20, wherein the tonoplast pyrophosphatase driven H+ pump gene transformed into the first plant is exogenous.

22. The method of Claim 20, wherein the second plant is a transgenic plant.

23. The method of Claim 20, wherein the second plant is a wild type plant.

24. The method of Claim 21, wherein said exogenous tonoplast pyrophosphate driven H+ pump gene is operably linked to a chimeric promoter.

25. The method of claim 24, wherein said exogenous tonoplast pyrophosphate driven H+ pump gene encodes AVP1.

26. A plant seed produced by the method of claim 21.

27. A progeny plant grown from the plant seed of claim 26.

28. The method of claim 22, wherein the first and second plants are from the species *A. thaliana*.

29. The method of claim 22, wherein the first and second plants are from the species *Nicotinia tabacum*.

30. The method of claim 22, wherein the second plant has been transformed with a polynucleotide sequence comprising an exogenous tonoplast pyrophosphatase driven H+ pump gene operably linked to a promoter.

31. A plant seed produced by the method of claim 22.

32. A progeny plant grown from the plant seed of claim 31.

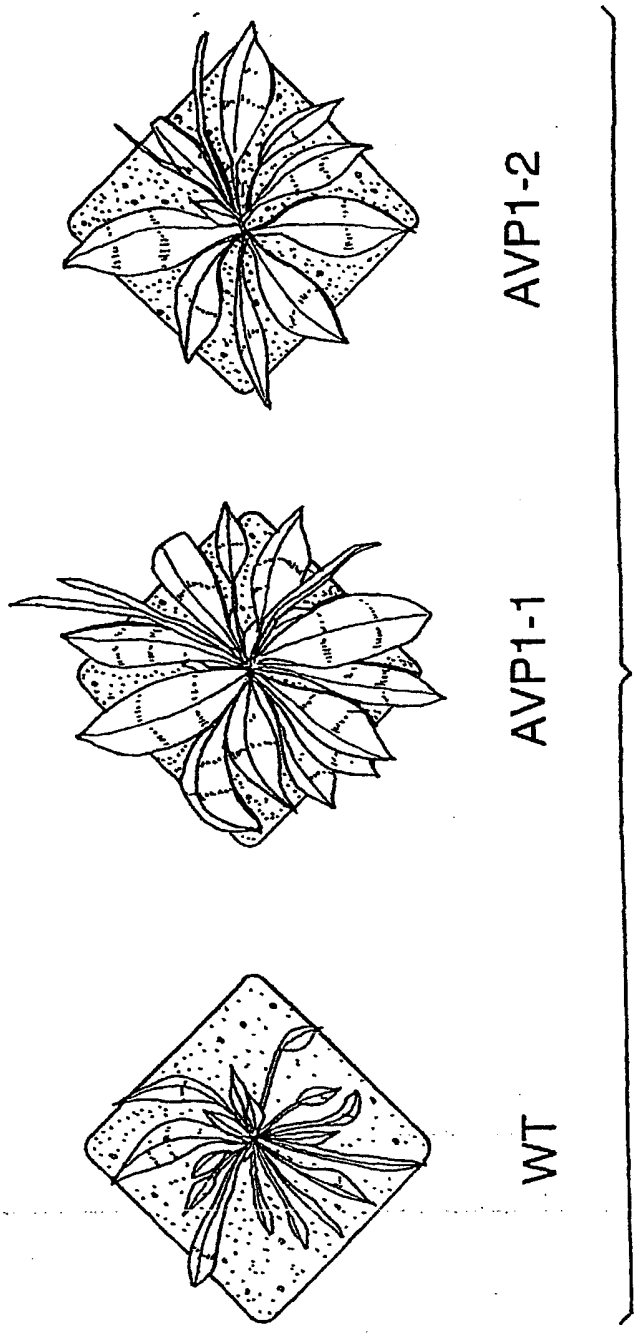


FIG. 1A

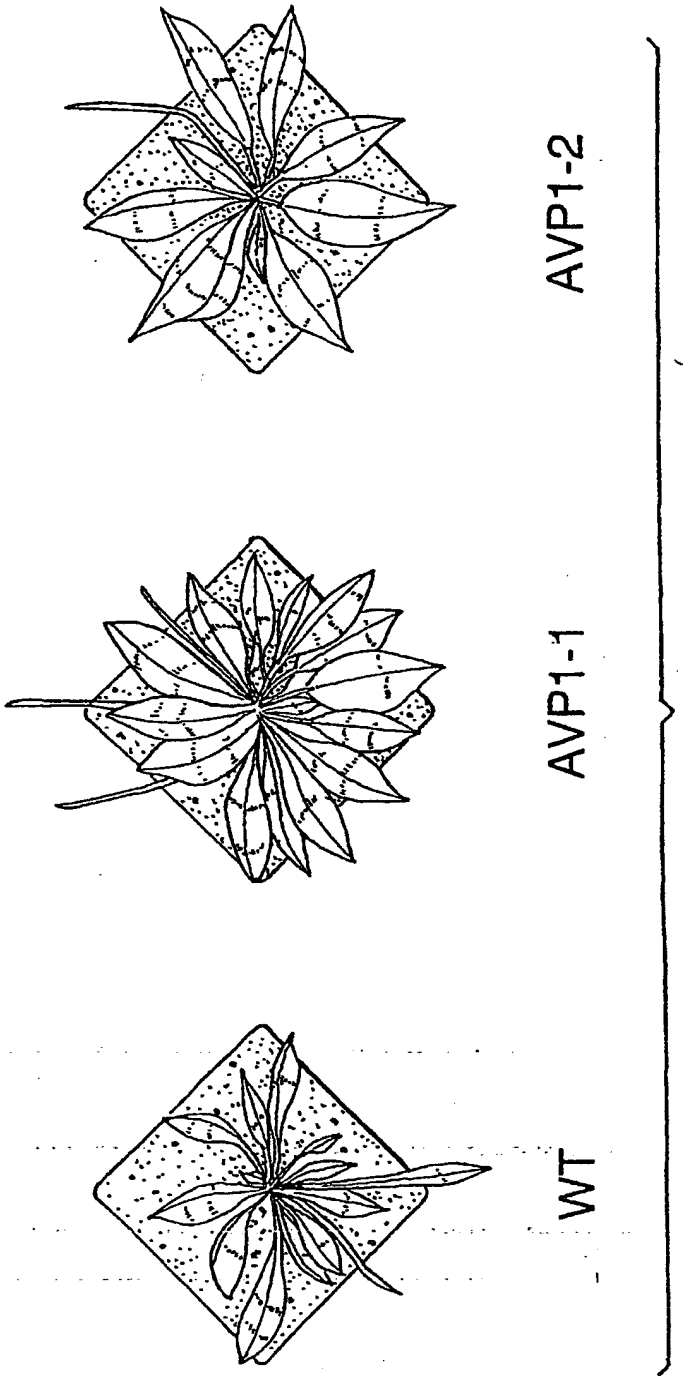


FIG. 1B

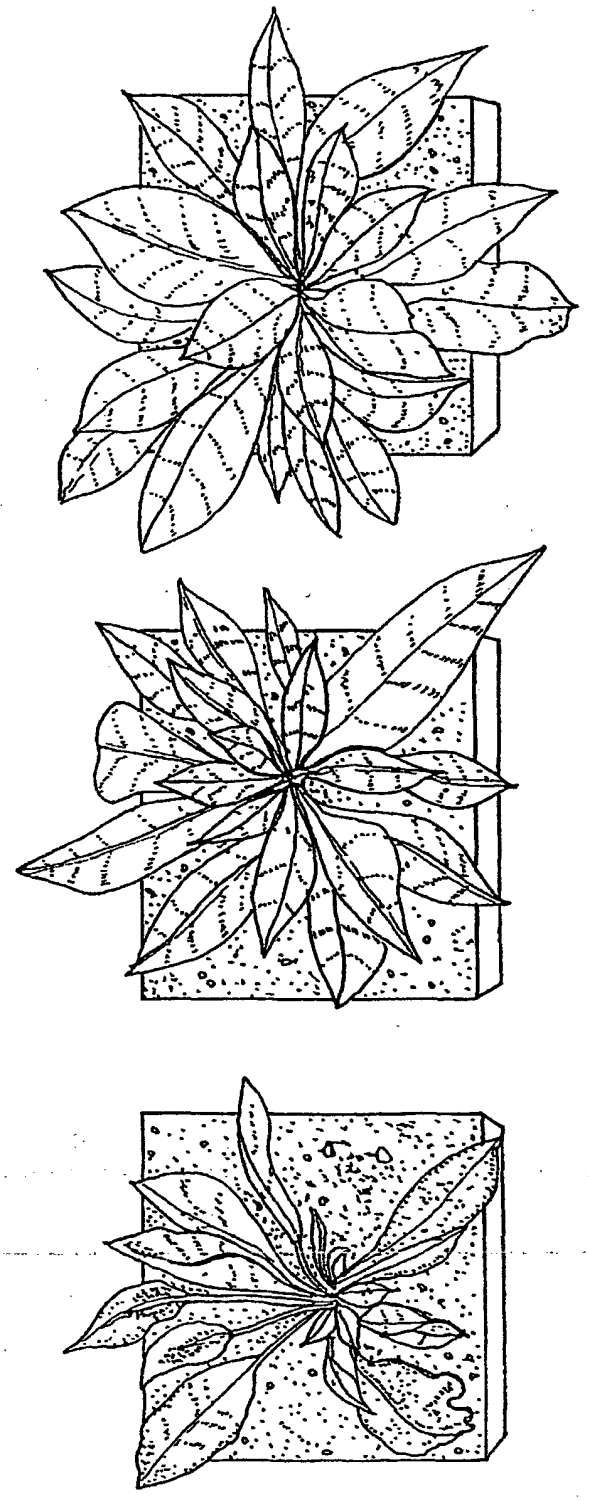


FIG. 2

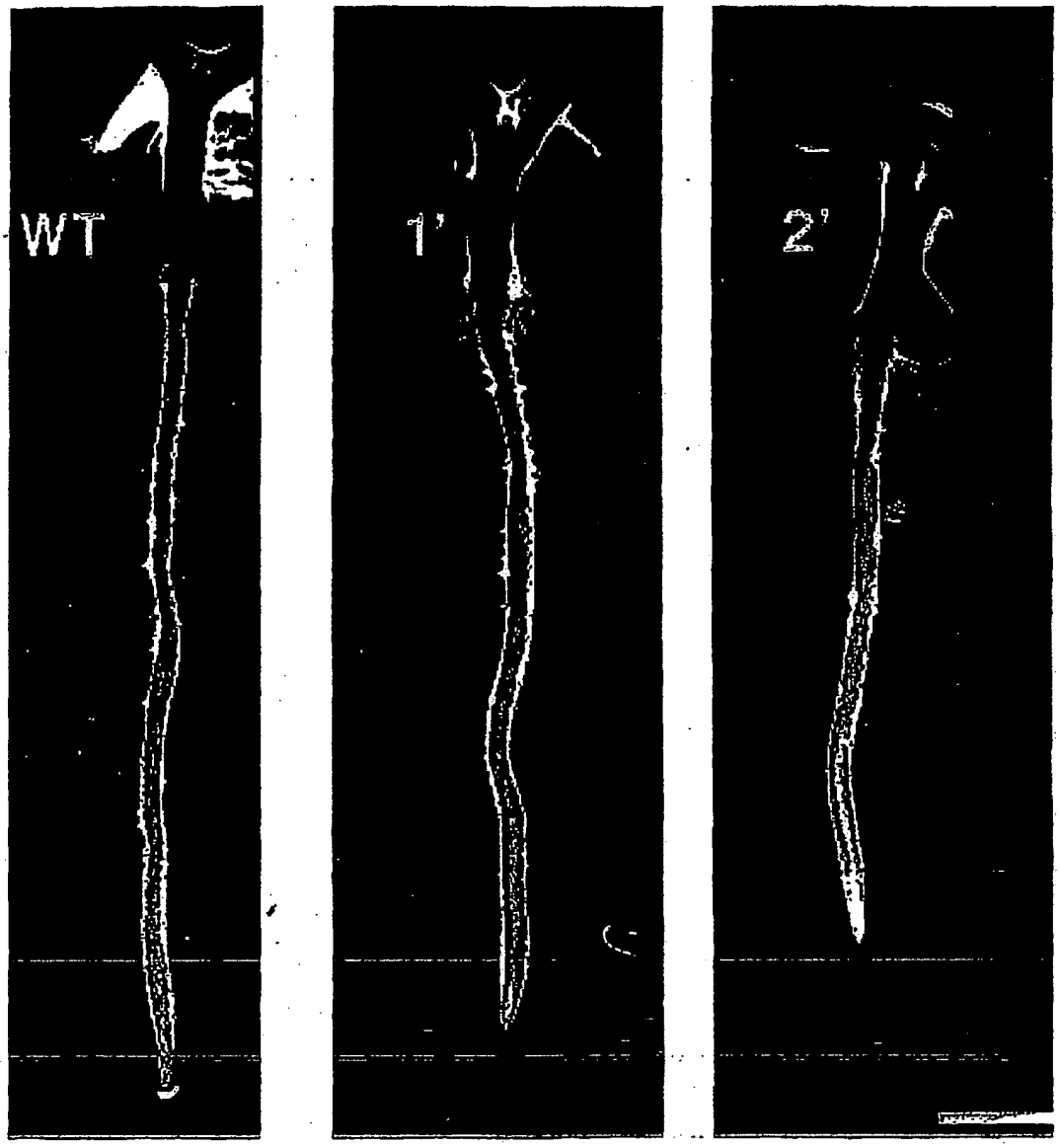


FIG. 3B(1) FIG. 3B(2) FIG. 3B(3)

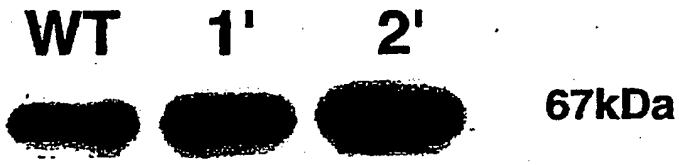


FIG. 4

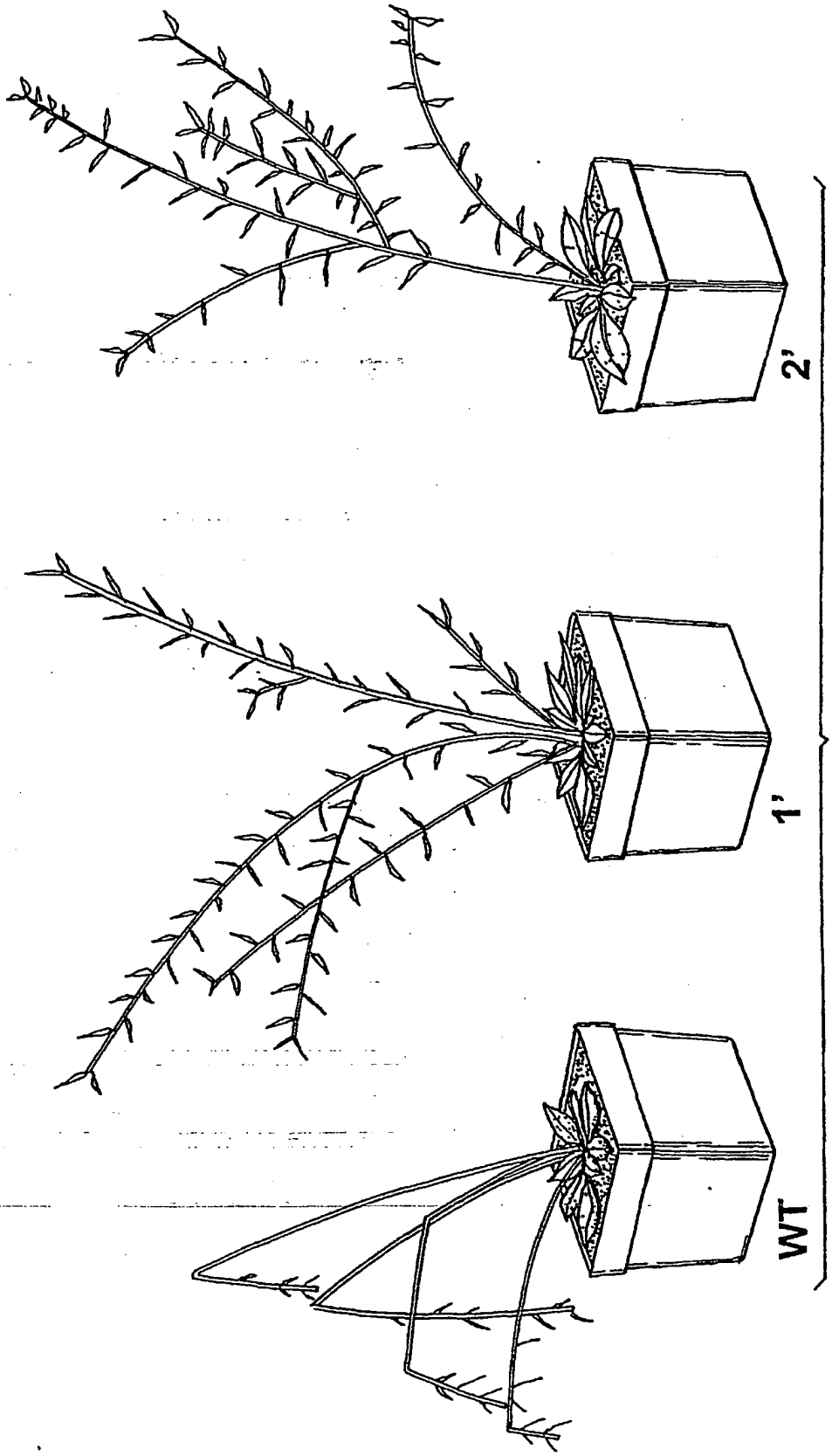


FIG. 5

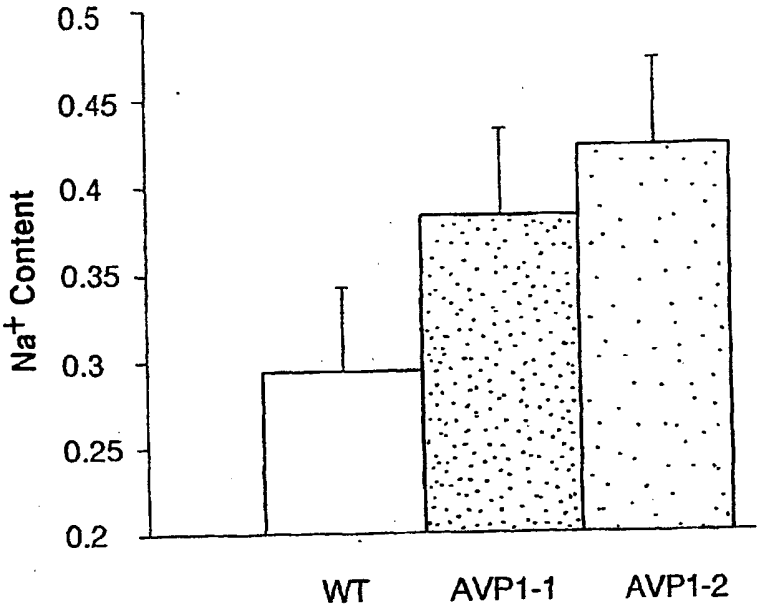

0.5
0.45
0.4
0.35
0.3
0.25
0.2
Na^+ Content
WT
AVP1-1
AVP1-2

FIG. 6A

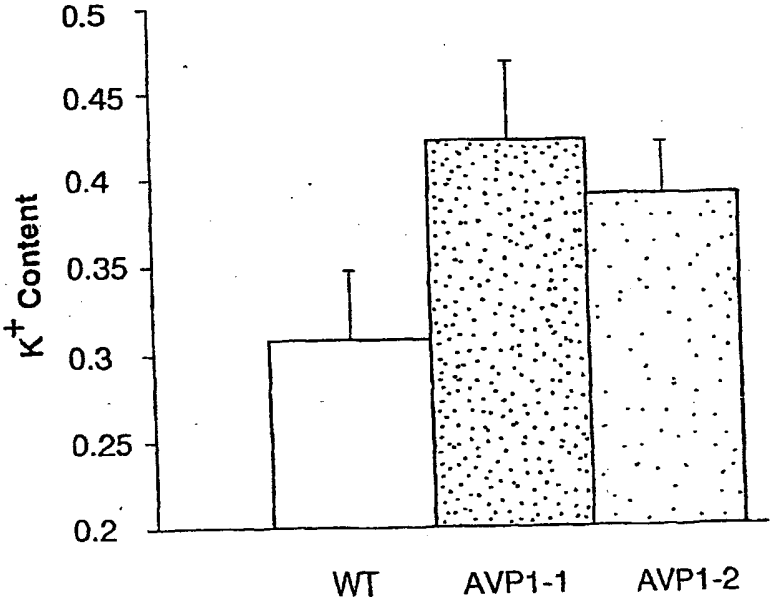

0.5
0.45
0.4
0.35
0.3
0.25
0.2
K^+ Content
WT
AVP1-1
AVP1-2

FIG. 6B

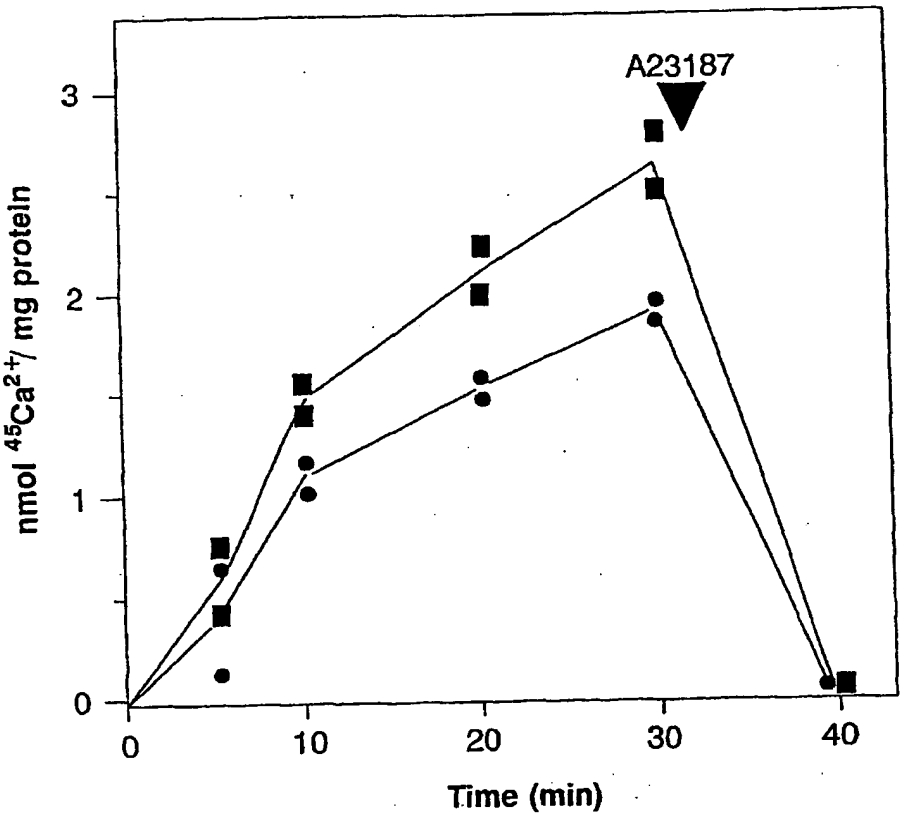


FIG. 6C

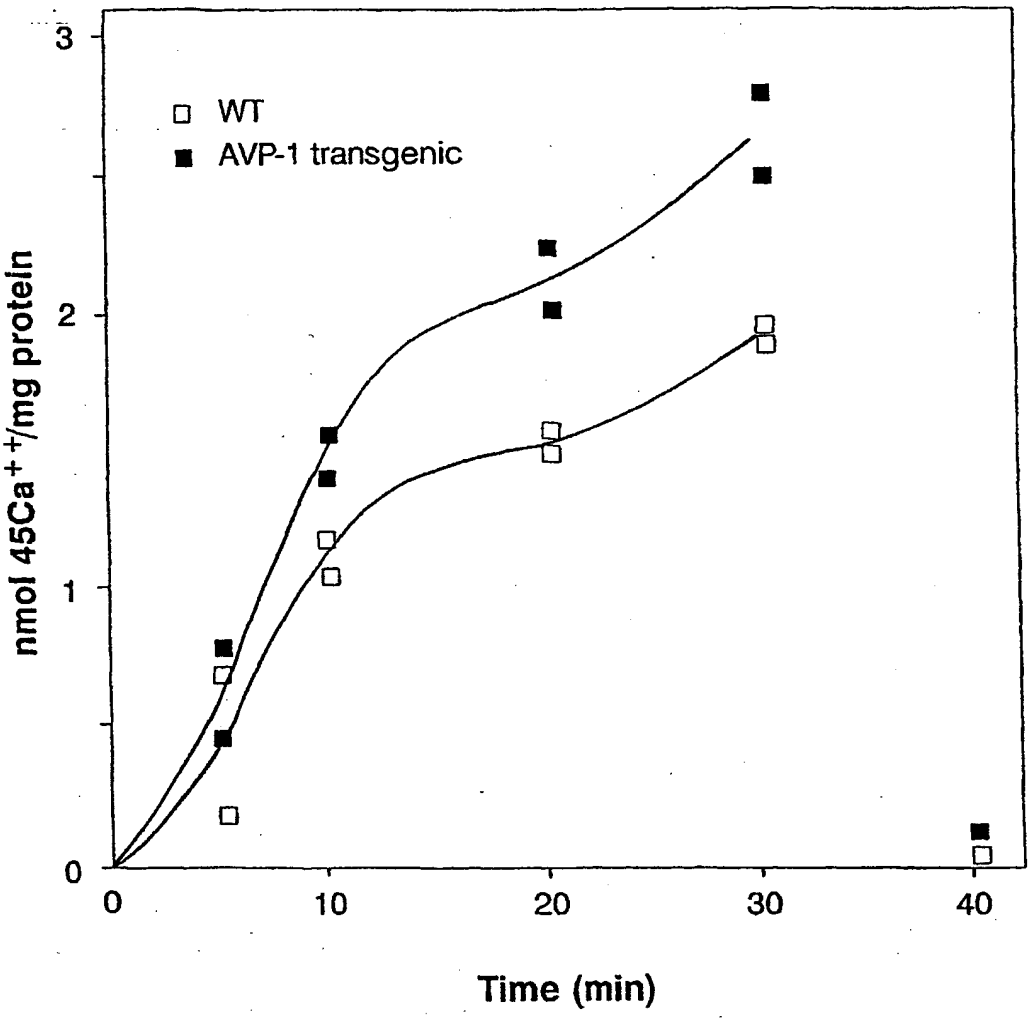

WT
AVP-1 transgenic
nmol 45Ca++/mg protein
Time (min)
0
1
2
3
0
10
20
30
40

FIG. 7

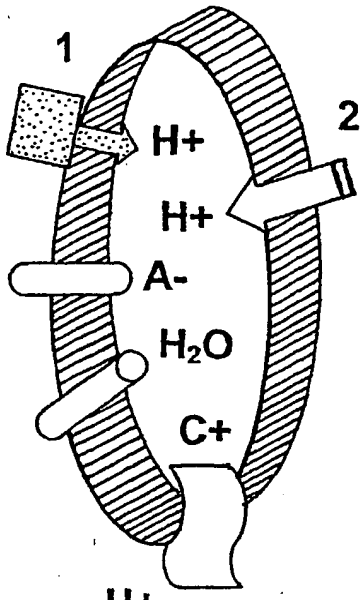


FIG. 8A

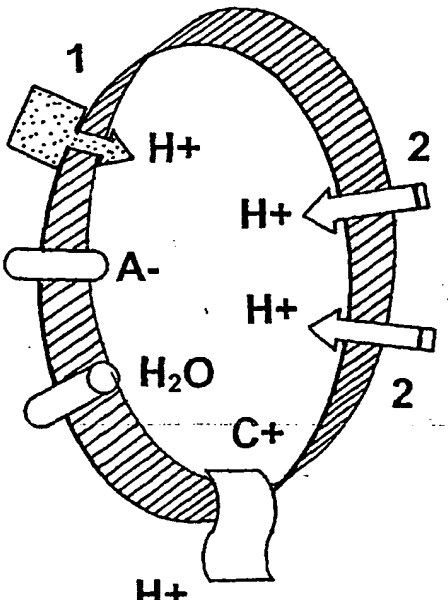
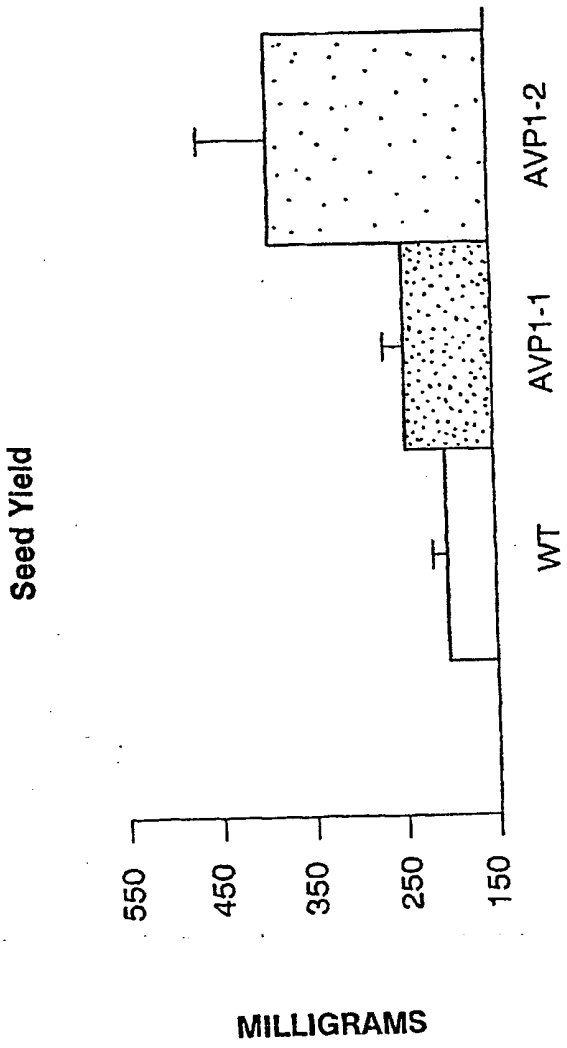
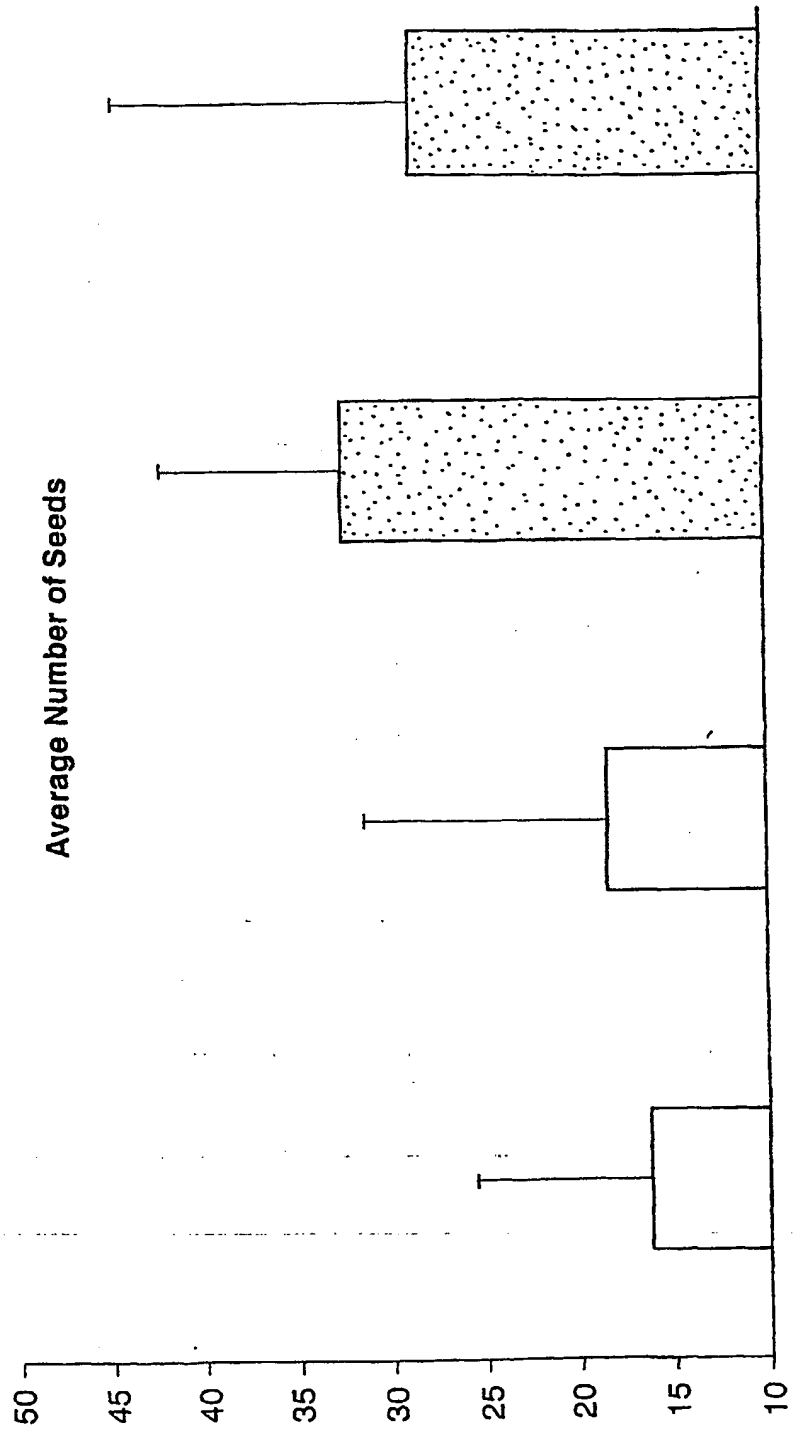


FIG. 8B



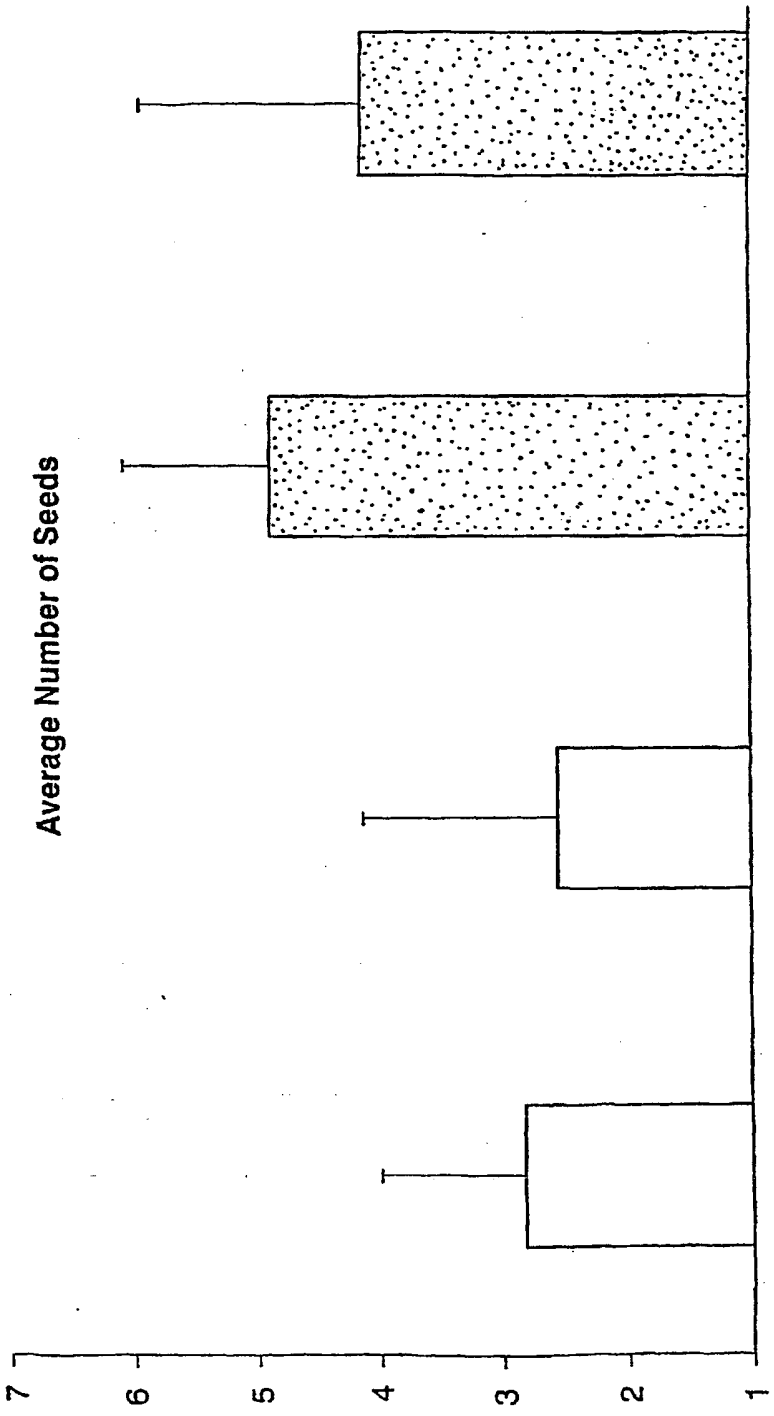
Wild type, *AVP1-1* and *AVP1-2* transgenic plants were grown in a 16 hour light / 8 hour dark cycle for two months. Seeds were harvest and weighted. Values are mean +/-SD (n=7).

FIG. 9



Pollen from wild type plants were used for pollination of transgenic *AVP1-1* and *AVP1-2* lines (white bars). Pollen from the above transgenic plants was used for pollination of wild type plants (grey bars). Values are means +/- SD (n=10).

FIG. 10A



Pollen from wild type plants were used for pollination of transgenic *AVP1-1* and *AVP1-2* lines (white bars). Pollen from the above transgenic plants was used for pollination of wild type plants (grey bars). Values are means +/- SD (n=10).

FIG. 10B

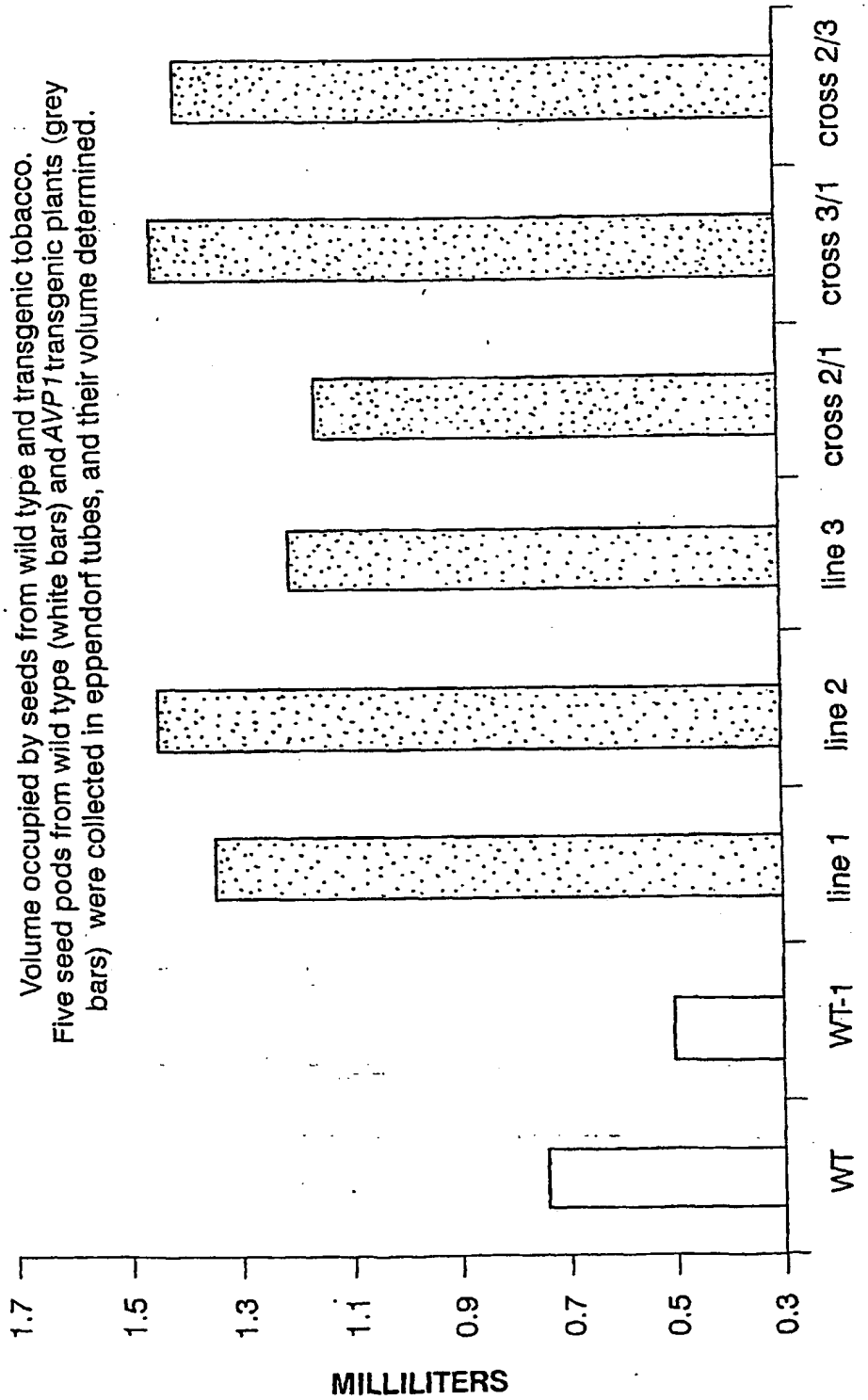

Volume occupied by seeds from wild type and transgenic tobacco.
Five seed pods from wild type (white bars) and AVP1 transgenic plants (grey bars) were collected in eppendorf tubes, and their volume determined.
1.7
1.5
1.3
1.1
0.9
0.7
0.5
0.3
MILLILITERS
WT
WT-1
line 1
line 2
line 3
cross 2/1
cross 3/1
cross 2/3
Volume of seeds in 5 tobacco pods

FIG. 11

FIG. 12A

FIG. 12B

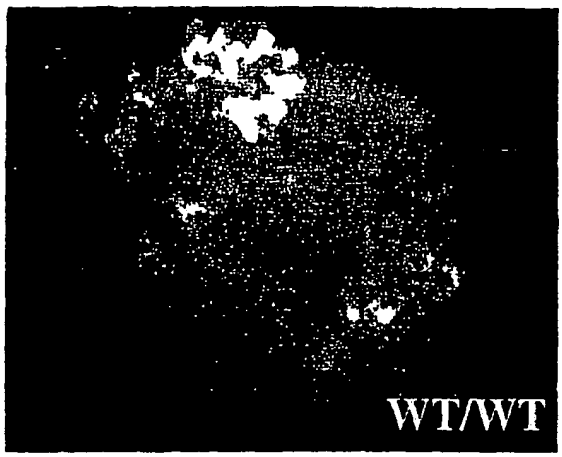


FIG. 13A

FIG. 13B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/41806

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C12N 5/04, 5/10; 15/82; A01H 1/00, 5/00
US CL : 435/419, 468; 800/260, 278, 298
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/419, 468; 800/260, 278, 298

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,E --- Y,E	GAXIOLA et al. Drought- and salt-tolerant plants result from overexpression of the AVP1 H+-pump. Proc. Natl. Acad. Sci. USA. 25 September 2001, Vol. 98, No. 20, pages 11444-11449, especially page 11445 Figure 1 and page 11446 Figure 3, for both X and Y.	1-7, 9-10, 12-18 --- 8, 11, 19-32
Y	GAXIOLA et al. The Arabidopsis thaliana proton transporters, AtNhx1 and Avp1, can function in cation detoxification in yeast. Proc. Natl. Acad. Sci. USA. February 1999, Vol. 96, pages 1480-1485, especially page 1481 Figure 1.	1-32
Y	SARAFIAN et al. Molecular cloning and sequence of cDNA encoding the pyrophosphate-energized vacuolar membrane proton pump of Arabidopsis thaliana. Proc. Natl. Acad. Sci. USA. March 1992, Vol. 89, pages 1775-1779, especially page 1777 Figure 2.	1-32

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 October 2001 (22.10.2001)	19 DEC 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Cynthia Collins Telephone No. (703) 308-0196

Janice Ford for

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/41806

Continuation of B. FIELDS SEARCHED Item 3:
WEST/STN (AGRICOLA, BIOSIS, CABA, CAPLUS, MEDLINE) search terms: inventor name, tonoplast pyrophosphate driven proton pump, AVP1, transgenic plant, stress tolerance, vacuolar pyrophosphatase.

SEQUENCE LISTING

<110> Andrews, William H.
Foster, Christopher A.
Fraser, Stephanie
Mohammadpour, Hamid

<120> METHODS AND COMPOSITIONS FOR MODULATING TELOMERASE REVERSE TRANSCRIPTASE (TERT) EXPRESSION

<130> SIER-003WO

<150> 60/227,682
<151> 2000-08-24

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gtgaggcact gtccccggg 1519

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/26039

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C12Q 3/00, 1/68; C12P 21/04; C12N 15/00, 5/00; C07H 21/04
US CL : 435/3, 6, 7.21, 320.1, 325; 514/44; 424/93.1; 536/ 24.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/3, 6, 7.21, 320.1, 325; 514/44; 424/93.1; 536/ 24.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE, CAPLUS, USPATFUL, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HORIKAWA, I. et al. Cloning and characterization of the promoter region of Human Telomerase. Cancer Research. 15 February 1999, Vol. 59, pages 826-830, see entire document.	1-22
A	WICK, M. et al. Genomic organization and promoter characterization of the gene encoding the human telomerase reverse transcriptase (hTERT). Gene. 1999, Vol. 232, pages 97-106, see entire document.	1-22
A	CROWE, D.L. et al. E2F-1 represses transcription of the human telomerase reverse transcriptase gene. Nucleic Acid Research. 2001, Vol. 29, No. 13, pages 2789-2794, see the entire document.	1-22

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search
06 November 2001 (06.11.2001)

Date of mailing of the international search report
31 DEC 2001

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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Authorized officer
Ram R. Shukla

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/26039

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-22

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/26039

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-22, drawn to a method of enhancing expression of TERT in an expression system comprising a Myc Repeat region in vivo and ex vivo.

Group II, claim(s) 23-33, drawn to a method of decreasing telomerase expression in a cell ex vivo and in vivo.

Group III, claim(s) 34-41, drawn to a nucleic acid comprising a nucleotide sequence that is same or identical to Myc repeat region.

Group IV, claim(s) 42-45, drawn to a double stranded DNA decoy sequence comprising a Myc repeat region.

Group V, claim(s) 46-49, drawn to a method of screening for agents that inhibit Myc repeat repression of TERT transcription.

Group VI, claim(s) 50 and 51, drawn to a mammalian cell comprising a telomerase gene that comprises a deletion of Myc repeat region.

Group VII, claim(s) 52, drawn to a method of producing a mammalian antibody.

The inventions listed as Groups I-VII do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The invention of group I requires an agent that enhances TERT expression whereas the method of group II requires an agent that inhibits TERT expression. Therefore the inventions of groups I and II lack the same special technical feature.

The invention of group III is a nucleic acid sequence that is identical to Myc repeat region and would include nucleic acid sequences that would not function as Myc repeat region and therefore, it lacks the same technical feature as groups I and II.

The invention of group IV is a double stranded decoy which is not required for the methods of groups I-II and therefore, lacks the same technical feature as that of groups I-II. Furthermore, its structure is distinct from that of the nucleic acid of group III.

The invention of group V lacks the same special technical feature because the steps of groups I-III can not be used to practice this method and this method can be used for making the compositions of groups III and IV.

The invention of group VI is drawn to a mammalian cell that can not be made by the methods of the groups I-II and V and its structure is distinct from that of the the compositions of groups III and IV.

The invention of group VII is for producing an antibody and its steps can be used for practicing the methods of groups I and II or vice versa. Furthermore, the compositions of groups III, IV, and V can not be used in this method.